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Choline and one-carbon metabolite response to egg, beef and fish among healthy young men: A short-term randomized clinical study

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SUMMARY

Background: Homeostatic mechanisms that regulate long-term circulating concentrations of choline and one-carbon metabolites can obscure the relationship between diet and nutritional response. As such, we sought to determine the acute response of one-carbon metabolites to animal source foods enriched in one-carbon nutrients.

Methods: As part of a crossover feeding trial with one-week washout intervals, healthy young men (n = 40) were randomized to animal food sources of choline (eggs, beef and fish) and a fruit control. A panel of one-carbon metabolites was measured in blood and urine samples collected at baseline and throughout the 6-h study period.

Results: Consumption of the test foods yielded 1.1–2.7 times higher ($P < 0.0001$) maximum peak circulating concentrations of choline, betaine, dimethylglycine (DMG) and methionine relative to the fruit control. Similarly, urinary excretion was 1.5–2.6 times higher ($P < 0.0001$) for these same metabolites across the 6-h study period. Of the test foods, eggs had the greatest effects on plasma choline and betaine, while fish had the greatest effects on plasma methionine. Nutritional response across the study period

Abbreviations: BMI, Body mass index; Cr, Creatinine; DMG, Dimethylglycine; FMO3, Flavin-containing monooxygenase 3; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; TMAO, Trimethylamine-N-oxide..

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was modified by time with a delay in peak plasma concentrations of choline and betaine after egg consumption. In addition, the *FMO3* G472A genotype (rs 2266782) modified plasma DMG, urinary trimethylamine-*N*-oxide and urinary methionine responses to the test foods ($P < 0.05$).

Conclusion: Consumption of animal source foods of choline improves circulating concentrations of choline and other one-carbon metabolites in a manner that is influenced by time and the *FMO3* G472A genotype.

This trial was registered at clinicaltrials.gov as NCT02558673.

1. Introduction

The one-carbon metabolic network consists of reactions involved in the transfer of one-carbon units. This network is important for cellular methylation whereby a methyl group is donated by *S*-adenosylmethionine (SAM), the universal methyl donor, to produce an array of methylated products including those involved in energy production, lipid metabolism and gene expression [1]. Several micronutrients participate in one-carbon metabolism including folate and betaine, the oxidized derivative of choline. Donation of a methyl group by betaine to homocysteine generates dimethylglycine (DMG) and methionine [2], the latter of which can be adenosylated to SAM, a universal methyl donor in cellular methylation.

One-carbon nutrients are found in both animal and plant food products. Animal foods such as eggs and beef are particularly good sources of choline and increased consumption of these foods is recommended as a means to improve choline status [3]. However, previous reports have shown that blood biomarkers of choline metabolism/status are not associated with dietary choline or betaine intake [4,5]. This lack of relationship between diet and nutritional response likely arises from the rapid tissue uptake of orally consumed choline [6] and the engagement of homeostatic mechanisms that regulate circulating concentrations of choline metabolites [7].

Thus, to better understand and quantify the effect of animal foods enriched in choline (and other methyl nutrients) on nutritional response, we examined the changes in circulating and urinary one-carbon metabolites immediately following the consumption of eggs, beef and fish, which were administered to young men as part of a randomized crossover feeding study. Furthermore, men were genotyped for the *FMO3* G472A (rs 2266782) single nucleotide polymorphism [8], a functional variant that impairs the conversion of trimethylamine, a derivative of choline generated in the gastrointestinal tract, to trimethylamine-*N*-oxide (TMAO) [9].

2. Methods and materials

2.1. Participants

Forty healthy men of age 21–50 y with a body mass index (BMI) of 20–29.9 kg/m^2 were recruited from the Cornell University's Ithaca campus and surrounding area from May to June 2014 as previously described [8]. A sample size of $n = 40$ was used to detect a 10% difference of plasma choline concentration at $\alpha < 0.05$ and $\beta = 0.8$ for a within-subject design. Participants were enrolled into the study contingent upon good health assessed by blood chemistry profile, cell count and health history questionnaire. Men of age >50 y, BMI $\geq$ 30 kg/m^2, women, vegetarians, smokers and individuals with gastrointestinal diseases or complaints, chronic illnesses or other metabolic diseases (including trimethylaminuria), abnormal blood chemistry values, and those taking nutritional

supplements, antibiotics or probiotics within 2 months of recruitment were excluded from the study. Written informed consent was obtained from all participants. The protocol was approved by the Institutional Review Board for Human Study Participants at Cornell University (Protocol ID#: 1403004534).

2.2. Study design

As part of a crossover feeding study [8], participants were randomized to three test foods: (i) eggs (3 whole hard-boiled; Wegmans); (ii) beef (6 ounces Philly-Gourmet Beef Patties, 100% Pure; Tops); and (iii) fish (6 ounces cod fillet; Tops); as well as the fruit control (2 single-serve packages of Mott's natural applesauce; Tops). Figure 1A illustrates a schematic of the study design and the participant flow through each stage of the randomized trial. The order of the test foods for each participant was determined by random number generator (random.org) and assigned by a study investigator. Each test food was administered in a single day separated by a 1-week washout period. All foods were prepared the morning of testing in the Human Metabolic Research Unit (HMRU) kitchen at Cornell University.

2.3. Protocol and sample collection

The experimental protocol was previously described in [8]. Figure 1B shows the study procedure. Briefly, participants arrived in the fasted state at the HMRU between 0700 and 1000 for each of the four

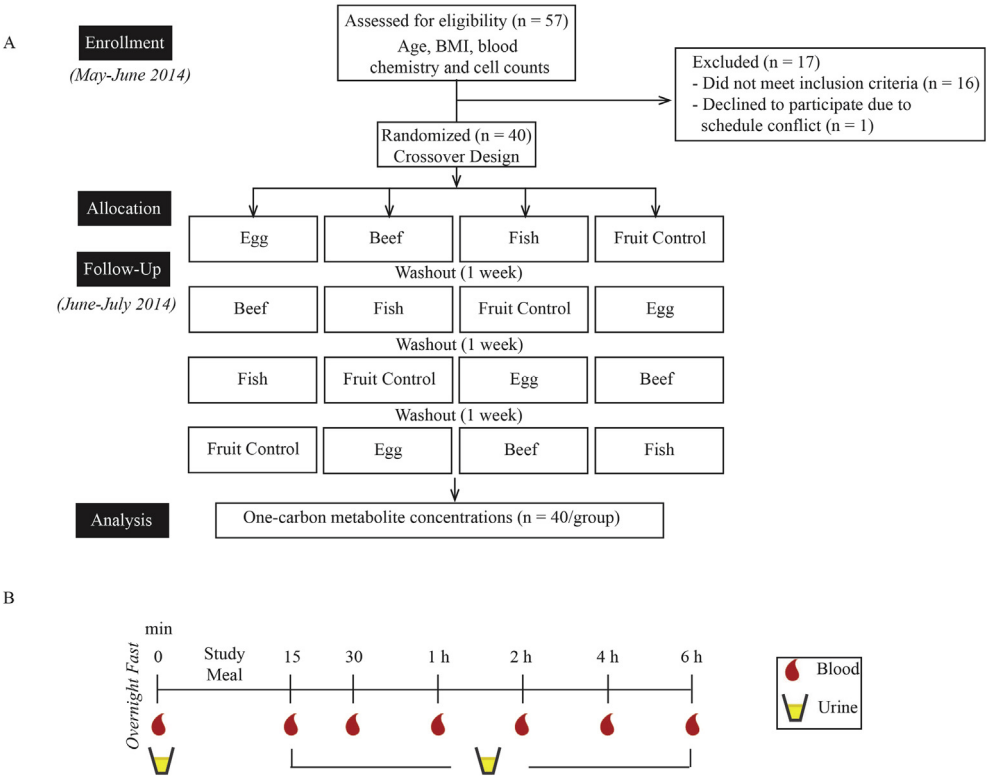

Fig. 1. A schematic of the study design including the participant flow through each stage of the randomized trial (A) and the study procedure (B).

visits, which were separated by one-week washout periods. A baseline blood sample was collected by standard venipuncture, and participants provided their baseline urine.

Participants then consumed a randomly allocated test food with one cup of water within 15 min. Following consumption of the test meal, serial blood samples were collected at 15, 30 min, 1, 2, 4 and 6-h, and processed for plasma [10–12]. Pooled urine was also collected throughout the 6-h study period. Participants refrained from consuming foods and beverages (other than water) outside those provided by study personnel. All samples were distributed into storage vials, de-identified and stored at −80 °C.

2.4. Food content and biomarker measurements

One-carbon metabolites (choline, betaine, DMG and methionine) were measured in study foods, plasma and urine by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [13,14] with modifications [10]. Metabolite concentrations in urine were adjusted for creatinine (Cr), which was measured using the Dimension Xpand chemistry analyzer (Siemens Healthcare Diagnostic) in the Human Nutritional Chemistry Service Laboratory at Cornell University.

2.5. Genotyping

DNA was extracted from buffy coat and the *FMO3* G472A (rs 2266782) variant [15] was genotyped with a fluorescent Taqman SNP genotyping assay kit (Thermo Scientific) using the LightCycler® 480 real-time PCR (Roche) according to the manufacturer's protocol.

2.6. Statistical analyses

Data were available for all forty men at every time point and statistical analyses were conducted in SAS (Version 9.3, SAS Institute). Two-way repeated measures analysis of variance (ANOVA) using the PROC MIXED model procedure was used to determine the effect of the test food, time and test food-by-time interaction on metabolite concentrations in plasma and urine. A significant interaction term was followed by one-way repeated measures ANOVA and Tukey–Kramer post-hoc test to describe differences between meals at each time point. The maximum peak concentration of a given metabolite in response to the test foods was compared using one-way ANOVA and Tukey–Kramer post-hoc test.

Possible covariates considered in our mixed models included age, BMI and study session order. Notably, we had previously genotyped these men for the FMO3 G472A single nucleotide polymorphism. When included as a covariate in our models in the current study, a significant influence of the *FMO3* G472A genotype was detected on some of the response variables. We therefore retained this covariate when it achieved a statistical significance of $P < 0.05$. To better understand the effects of the *FMO3* G472A genotype on the one-carbon metabolites, the genotype was entered into the models as an independent variable. Significant differences were reported at a False Discovery Rate adjusted $P < 0.05$. All data are expressed as means ± SEM.

3. Results

3.1. Participant characteristics

Healthy men ($n = 40$) with a mean age of 27.8 ± 1.0 y and BMI of 24.2 ± 0.4 kg/m^2 completed the study as previously described [8]. The distribution of the *FMO3* G472A genotype was 14 wild-type GG, 22 heterozygous GA and 4 homozygous AA.

3.2. Food content

Food concentrations of total choline, betaine and methionine in egg, beef, fish and fruit are shown in Table 1. DMG was not detected in any of the meals.

Table 1
Food content of total choline (sum of free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, sphingomyelin), betaine and methionine in egg, beef, fish and fruit. DMG was not detected in any of the foods. Values within rows with different letter superscripts show a significant effect of the test food (one-way ANOVA, Tukey–Kramer post-hoc test). Values are mean ± SEM, each meal has 5 replicates.

Food content (mg)	Fruit	Egg	Beef	Fish	P value
Total Choline	3.8 ± 0.1^{a}	479 ± 16^{c}	132 ± 7.0^{b}	161 ± 0.6^{b}	<0.0001
Betaine	0.0 ± 0.0^{a}	0.9 ± 0.0^{a}	12.7 ± 0.3^{b}	12.4 ± 0.2^{b}	<0.0001
Methionine	0.0 ± 0.0^{a}	7.5 ± 0.4^{c}	3.0 ± 0.1^{b}	6.4 ± 0.2^{c}	<0.0001

3.3. Plasma free choline, betaine, DMG and methionine

Plasma one-carbon metabolites showed a significant effect of the test food ($P < 0.05$), time ($P < 0.0001$) and test food-by-time interaction ($P < 0.0001$). Consumption of the animal food sources of choline yielded higher ($P < 0.0001$) circulating concentrations of all one-carbon metabolites across the study period (Fig. 2). Relative to the fruit control, plasma free choline was 1.6-times higher after egg, 1.4-times higher after fish, and 1.2-times higher after beef ($P < 0.0001$); plasma betaine was 1.2-times higher after egg, and 1.1-times higher after fish and after beef ($P < 0.0001$); plasma DMG was 1.5-times higher after fish, and 1.3-times higher after egg and after beef ($P < 0.0001$); and plasma methionine was 2.7-times higher after fish, and 1.8-times higher after egg and after beef ($P < 0.0001$) (Table 2). Plasma concentrations of *S*-adenosylhomocysteine (SAH), the product of SAM-dependent methylation reactions, also differed among the test foods at study-end with fish yielding 14% higher concentrations than the fruit control ($P = 0.007$; data not shown).

In terms of trajectory over time, plasma free choline and betaine peaked 1-h after fish and beef consumption and 2-h after egg consumption. The decline in plasma choline and betaine through time was attenuated after egg consumption as compared to the other test foods which did not differ from baseline values at study-end. Plasma DMG and methionine peaked at 2-h in response to all test foods. DMG remained elevated ($P < 0.0001$) throughout the study period, while methionine remained elevated in response to fish, egg and beef at 4-h (fish > egg and beef > fruit) and in response to fish and egg at 6-h ($P < 0.0001$).

3.4. Urinary free choline, betaine, DMG and methionine

Similar to plasma, significant effects of the test food ($P < 0.0001$), time ($P < 0.0001$) and test food-by-time interaction ($P < 0.0001$) were detected (Table 3). Relative to the fruit control, urinary free choline was 1.8-times higher after egg, 1.6-times higher after fish, and 1.1-times higher after beef ($P < 0.0001$); urinary betaine was 1.7-times higher after fish, 1.5-times higher after beef, and 1.2-times higher after egg ($P < 0.0001$); urinary DMG was 2.2-times higher after fish, 2-times higher after beef, and 1.6-times higher after egg ($P < 0.0001$); and urinary methionine was 2.6-times higher after fish, 1.6-times higher after egg, and 1.5-times higher after beef ($P < 0.0001$) (Table 3).

3.5. Genetic polymorphism

The *FMO3* G472A genotype interacted with the test foods and time ($P = 0.02$, 3-way interaction) to influence plasma DMG response (Fig. 3). Compared to the GG genotype, the AA genotype had 2-times higher plasma DMG, while the GA genotype had 1.3–1.4 times higher plasma DMG across the study period for egg, fish and fruit ($P = 0.009$). The *FMO3* G472A genotype also interacted with the test foods and time (urinary TMAO: $P = 0.04$, time-by-genotype interaction; urinary methionine: $P = 0.01$ time-by-genotype interaction; $P = 0.04$ 3-way interaction) to influence urinary excretion of TMAO and methionine (Table 4). The AA genotype had significantly lower TMAO after fish consumption compared to the GG genotype ($P = 0.04$), and the AA and GA genotypes had significantly lower methionine after beef consumption compared to the GG genotype ($P = 0.02$).

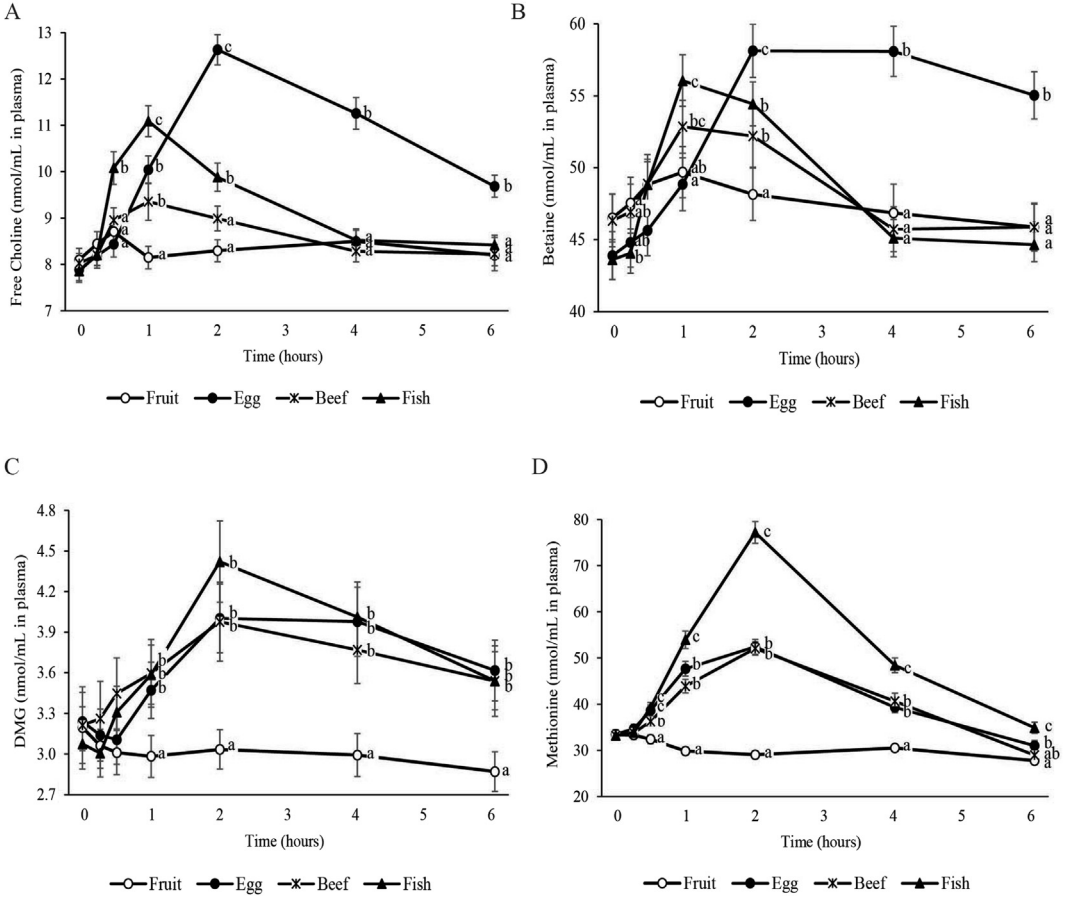

Fig. 2. Effect of the test foods on plasma concentrations of free choline, betaine, dimethylglycine (DMG) and methionine throughout the 6-h study period. Different letter superscripts show a significant effect of test food at each time point (one-way ANOVA, Tukey–Kramer post-hoc test). Values are mean ± SEM, n = 40.

Table 2
Effect of the test foods on plasma concentrations of choline, betaine, dimethylglycine (DMG) and methionine at maximum peak concentrations. The relative difference among the test foods at maximum peak concentrations was compared to the fruit control. Values within rows with different letter superscripts show a significant effect of the test food (one-way ANOVA, Tukey–Kramer post-hoc test). Values are mean ± SEM, n = 40.

	(nmol/mL plasma)	Fruit	Egg	Beef	Fish	P value
Choline	0 min	7.6 ± 0.2	7.4 ± 0.2	7.5 ± 0.2	7.4 ± 0.2	NS
	1-h	7.6 ± 0.2^{a}	9.5 ± 0.3^{b}	8.9 ± 0.4^{b}	10.6 ± 0.3^{c}	P < 0.0001
	2-h	7.8 ± 0.2^{a}	12.1 ± 0.3^{c}	8.5 ± 0.3^{a}	9.4 ± 0.3^{b}	P < 0.0001
	Relative Difference	1.0	1.6	1.2	1.4	
Betaine	0 min	47 ± 1.7	44 ± 1.7	46 ± 1.8	44 ± 1.4	NS
	1-h	50 ± 1.8^{ab}	49 ± 1.8^{a}	53 ± 1.8^{bc}	56 ± 1.8^{c}	P < 0.0001
	2-h	48 ± 1.8^{a}	58 ± 1.8^{c}	52 ± 2.2^{b}	54 ± 1.5^{b}	P < 0.0001
	Relative Difference	1.0	1.2	1.1	1.1	
DMG	0 min	3.2 ± 0.2	3.2 ± 0.2	3.2 ± 0.3	3.1 ± 0.2	NS
	2-h	3.0 ± 0.1^{a}	4.0 ± 0.3^{b}	4.0 ± 0.3^{b}	4.4 ± 0.3^{b}	P < 0.0001
	Relative Difference	1.0	1.3	1.3	1.5	
Methionine	0 min	33 ± 0.9	33 ± 1.0	33 ± 0.8	33 ± 0.9	NS
	2-h	29 ± 0.8^{a}	52 ± 1.6^{b}	52 ± 1.4^{b}	77 ± 2.3^{c}	P < 0.0001
	Relative Difference	1.0	1.8	1.8	2.7	

4. Discussion

The relationship between dietary choline intake and circulating concentrations of choline metabolites is obscured by rapid tissue uptake and the engagement of homeostatic mechanisms that maintain fasting concentrations within a narrow range. Thus to better understand the relationship between dietary choline intake and nutritional response, the current study examined blood and urinary biomarkers immediately after an acute intake of commonly consumed animal source foods (i.e., eggs, beef and fish). Two main findings emerged: (i) consumption of all three animal source foods increased circulating concentrations of the one-carbon metabolites in a manner that differed across time; and (ii) the *FMO3* G472A genotype was an important effect modifier of one-carbon nutritional response to animal source foods.

4.1. Consumption of animal source foods leads to acute changes in circulating choline and other one-carbon metabolites in a time-dependent manner

Consumption of the egg, beef and fish test foods as compared to the fruit control yielded higher circulating concentrations of choline, betaine, DMG and methionine. Since DMG was not present in the test meals, its rise in circulation after consumption of the animal source foods indicates that it was generated during oxidation of choline within the intestinal cell and/or liver. Eggs had the greatest effects on plasma choline and betaine, which exhibited a time course that differed from fish and meat. Plasma concentrations of choline peaked at 1-h for beef and fish, but not until 2-h for eggs. These time course differences in achievement of maximum peak concentrations may be due to the presence of different forms of choline in the test foods. The lipid-soluble forms of choline are more abundant in eggs (~220 mg) as compared to fish and beef (~30–70 mg) [16], and would be expected to enter the bloodstream at a slower rate than the water-soluble forms found in fish and beef.

Fish had the greatest effects on plasma and urinary methionine concentrations despite having a numerically lower food methionine content than eggs (Table 1). The substantial rise in methionine that occurred between the 1–2 h timeframe in fish, as compared to eggs, suggests that a portion of this methionine was generated endogenously. Notably, higher concentrations of plasma SAH were detected at study-end after fish consumption, as compared to the fruit control, suggesting that the methyl-group

Table 3
Effect of the test foods on urinary concentrations of choline, betaine, dimethylglycine (DMG) and methionine adjusted for creatinine (Cr) at study-baseline (0 min) and across the 6-h study period. The relative difference among the test foods across the 6-h study period was calculated as a ratio of the fruit control. Values within rows with different letter superscripts show a significant effect of the test food (one-way ANOVA, Tukey–Kramer post-hoc test). NS denotes not significant. Values are mean ± SEM, n = 40.

(nmol/mmol Cr in urine)	Time	Fruit	Egg	Beef	Fish	P value
Choline	0 min	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	NS
	Study (0–6 h)	2.1 ± 0.1^{a}	3.8 ± 0.2^{c}	2.3 ± 0.2^{a}	3.4 ± 0.2^{b}	P < 0.0001
	Relative Difference	1.0	1.8	1.1	1.6	
Betaine	0 min	5.3 ± 0.6	4.9 ± 0.5	4.9 ± 0.4	5.5 ± 0.6	NS
	Study (0–6 h)	7.3 ± 0.7^{a}	8.9 ± 0.9^{a}	10.9 ± 1.2^{b}	12.3 ± 1.3^{b}	P < 0.0001
	Relative Difference	1.0	1.2	1.5	1.7	
DMG	0 min	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.3	1.9 ± 0.2	NS
	Study (0–6 h)	2.6 ± 0.2^{a}	4.0 ± 0.3^{b}	5.0 ± 0.7^{bc}	5.7 ± 0.4^{c}	P < 0.0001
	Relative Difference	1.0	1.6	2.0	2.2	
Methionine	0 min	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	NS
	Study (0–6 h)	1.1 ± 0.1^{a}	1.7 ± 0.1^{b}	1.7 ± 0.1^{b}	2.8 ± 0.2^{c}	P < 0.0001
	Relative Difference	1.0	1.6	1.5	2.6	

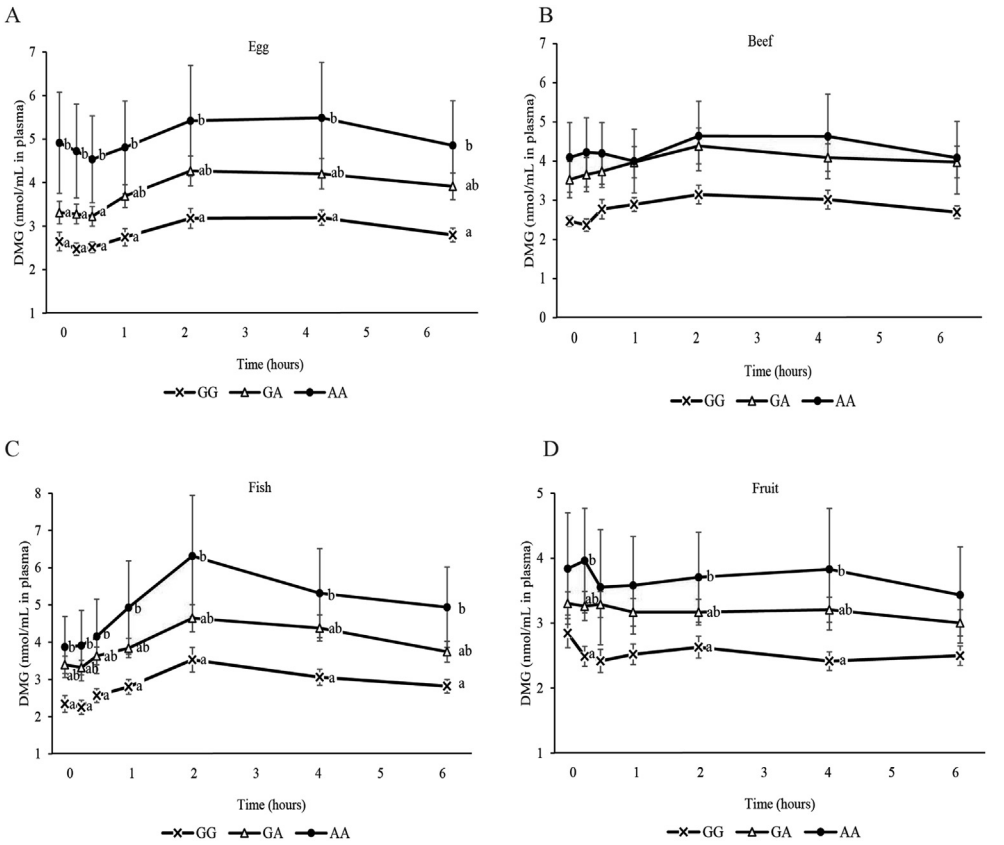

Fig. 3. Effect of the flavin-containing monooxygenase 3 (*FMO3*) G472A genotype on plasma dimethylglycine (DMG) concentrations before and after consumption of the test foods. Different letter superscripts show a significant effect of genotype at each time point for each test food (one-way ANOVA, Tukey–Kramer post-hoc test). Values are mean ± SEM; n = 40: 14 GG, 22 GA and 4 AA.

Table 4
Effect of the flavin-containing monooxygenase 3 (*FMO3*) G472A variant on urinary excretion of trimethylamine-*N*-oxide (TMAO) and methionine adjusted for creatinine (Cr) in response to the egg, beef, fish and fruit control. Values within rows with different letter superscripts show a significant effect of genotype at each time point (one-way ANOVA, Tukey–Kramer post-hoc test. NS denotes not significant. Values are mean ± SEM, n = 40.

(nmol/mmol Cr in urine) *FMO3* G472A		TMAO				Methionine			
		GG	GA	AA	P value	GG	GA	AA	P value
Fruit	0 min	27 ± 2.4	40 ± 11.5	29 ± 8.5	NS	1.1 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	NS
	Study (0–6 h)	38 ± 5.7	48 ± 9.7	28 ± 0.7	NS	1.2 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	NS
Egg	0 min	25 ± 3.1	29 ± 4.4	28 ± 9.5	NS	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	NS
	Study (0–6 h)	38 ± 5.8	35 ± 4.8	28 ± 5.4	NS	2.0 ± 0.2	1.5 ± 0.1	1.5 ± 0.2	NS
Beef	0 min	32 ± 6.0	32 ± 5.7	27 ± 5.2	NS	1.1 ± 0.1	0.9 ± 0.0	1.1 ± 0.4	NS
	Study (0–6 h)	40 ± 8.5	42 ± 11.2	25 ± 7.2	NS	2.1 ± 0.2^{a}	1.5 ± 0.1^{ab}	1.2 ± 0.3^{b}	0.004
Fish	0 min	38 ± 10.3	37 ± 5.4	25 ± 5.2	NS	1.1 ± 0.1	1.0 ± 0.0	0.9 ± 0.1	NS
	Study (0–6 h)	1590 ± 103^{a}	1475 ± 60^{ab}	1132 ± 120^{b}	0.05	3.4 ± 0.4	2.5 ± 0.2	2.2 ± 0.3	NS

associated with some of the methionine was subsequently donated by SAM within the 6-h time frame of this human study.

In general, changes in the urinary excretion of the one-carbon metabolites in response to the test foods paralleled those of plasma. Thus urine appears to be a viable medium for assessing the nutritional response of one-carbon metabolites to these foods. However, it is interesting to note that despite lower plasma betaine concentrations, urinary betaine excretion was greater for fish and beef as compared to eggs. This implies that food-derived betaine may belong to an endogenous pool that is eliminated more readily than betaine derived from the oxidation of choline.

4.2. FMO3 *G472A genotype modifies nutritional response to animal source foods*

An influential covariate in the current study was the *FMO3* G472A genotype. When assessed as an independent variable, men with the AA genotype excreted less TMAO than those with the GG genotype following the consumption of fish, a finding that is consistent with a reduced capacity for *N*-oxidation of trimethylamine among men with the variant allele [17]. Surprisingly, we also observed that men with the AA (versus GG) genotype excreted less methionine and had higher circulating DMG concentrations. Methionine and DMG are produced when betaine is used as a methyl donor to remethylate homocysteine to methionine. Higher plasma DMG among men with two copies of the variant allele suggests greater generation of methionine from betaine, while lower methionine excretion suggests greater retention of methionine. Thus, it seems that individuals with reduced capacity of the FMO3 enzyme generate and retain more methionine. Our findings align with other reports demonstrating metabolic functions of FMO3 that extend beyond its well-established role in the conversion of trimethylamine to TMAO [18–21].

5. Strengths and limitations

This is the first study to report the acute response of one-carbon metabolites to animal source foods enriched in choline among healthy young men. However, this study used a whole-food approach and therefore the effect of specific dietary components on metabolic response remains unknown. In addition, the study findings may not be generalizable to other populations such as those with intestinal disorders and chronic diseases.

6. Conclusion

We show that consumption of animal food sources deemed to be enriched in choline improve circulating concentrations of choline and other one-carbon metabolites. *FMO3* G472A genotype

appears to modify the nutritional response of these one-carbon metabolites but additional studies with larger sample sizes are needed to confirm these findings.

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Author contributions

CEC contributed to the study design, collected the data, conducted the statistical analyses, interpreted the data and prepared the manuscript. ST contributed to the study design, collected the data, and assisted in the statistical analyses and data interpretation. OVM, EB and JY provided technical assistance and contributed to the data collection. MAC conceived the study, contributed to the data interpretation and critically reviewed the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest. None of the funding sources had any role in the study design, sample analyses, data interpretation and manuscript preparation.

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